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ORIGINAL PAPER

Alteration of glutathione S-transferase properties during the development of *Micromelalopha troglodyta* larvae (Lepidoptera: Notodontidae)

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Abstract: *Micromelalopha troglodyta* (Graeser) is an important pest of poplar in China. Glutathione S-transferases (GSTs) are known to be responsible for adaptation mechanisms of *M. troglodyta*. The activities and kinetic constants of glutathione S-transferases in *M. troglodyta* were studied. Significant differences in glutathione S-transferase activity and kinetic characteristics were observed among five instars of *M. troglodyta* larvae. Furthermore, the inhibition of glutathione S-transferase activity in five instars by 24 inhibitors was conducted. The results show the inhibition of GST activity of different instars by 24 inhibitors was different. For GST activity in the 1st instar, chlorpyrifos, lambda-cyhalothrin, endosulfan, abamectin, fipronil and pyridaben were the best inhibitors tested, and for GST activity in the 2nd instar, tannic acid and quercetin were the most potent inhibitors tested, and for GST activity in the 3rd instar, the inhibitory effects of quercetin, chlorpyrifos and lambda-cyhalothrin were the highest, and for GST activity in the 4th instar, quercetin and lambda-cyhalothrin were the best inhibitors, and the inhibitory effect of phoxim was the highest for GST activity in the 5th instar. Our results show that glutathione S-transferases in different instars are qualitatively different in isozyme composition and thus different in sensitivity to inhibitors.

Keywords: glutathione S-transferases; *Micromelalopha troglodyta*; instar; kinetic characteristics; inhibitor

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Introduction

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a group of multifunctional detoxification enzymes catalyzing the conjugation of reduced glutathione (GSH) with electrophilic compound. When conjugated to GSH, potentially toxic substances become more water soluble and generally less toxic. Thus, glutathione-dependent conjugation has been regarded as an important detoxification mechanism in insect and mammals. Furthermore, GSTs have long been demonstrated to be involved in protection from oxidative damage and intracellular transport of hormones, endogenous metabolites and exogenous chemicals (Listowsky et al. 1988, Clark 1989, Rushmore & Pickett 1993). Glutathione S-transferases in lepidopterous insects metabolize various toxic allelochemicals, including α,β-unsaturated carbonyl compounds (e.g., trans-cinnamaldehyde), isothiocyanates (e.g., allyl isothiocyanate), and organothiocyanates (e.g., benzyl thiocyanate) (Wadleigh & Yu 1988, Yu & Abo-Elghar 2000). In insects, GSTs provide an important defense mechanism against insecticides (Motoyama & Dauterman 1980) as well as plant alleochemicals (Yu 1986). Therefore, GSTs play an important role in insect.

Micromelalopha troglodyta (Graeser) (Lepidoptera: Notodontidae), is an important pest of poplar in China, causing damage by direct feeding, and insecticides are widely used for its control. Detoxification enzymes are known to be responsible for adaptation mechanisms, among these enzymes are glutathione S-transferases. Presently very little is known about the biochemical properties of GSTs in *M. troglodyta*. In the present study, the development change of GSTs from *M. troglodyta* larvae is reported. These results may contribute to the understanding of the sensitivity of different instars of *M. troglodyta* larvae to pesticides and help determine their role in the metabolism and excretion of pesticides. Furthermore, another purpose of this study was to find some potential GST inhibitors which might be

used as synergists to enhance the toxicity of insecticides.

Materials and Methods

Chemicals

1-chloro-2,4-dinitrobenzene (CDNB, ≥99%), reduced glutathione (GSH, ≥99%), tannic acid (>99%) and 2-tridecanone (>99%) were purchased from Sigma Chemical Co. (St. Louis, MO), phenylmethylsulfonyl (PMSF, >99%) from Merk (Darmstadt, Germany), dithiothreitol (DTT, 99%) from Promega (Madison, WI), quercetin (99%) from Shanghai chemical stock (Shanghai, China). triazophos (92.0%) from Jiangxi Kaifeng Chemical Co., Ltd (Jiangxi, China), malathion (95.0%) from Hebei Shiji Pesticide Co., Ltd (Hebei, China), chlorpyrifos (97.0%) from Dow AgroSciences LLC (Indiana, USA), phoxim (99.0%) and profenofos (90.0%) from Tianjin Pesticide Co., Ltd (Tianjin, China), Omethoate (92.0%) from Hangzhou Qingfeng Agrochemicals Co., Ltd (Zhejiang, China), methomyl (98.0%) from Hubei Sanongda Co., Ltd (Hubei, China), fenpropathrin (92.0%) and beta-cypermethrin (97.0%) from Shandong Dacheng Pesticide Co., Ltd (Shandong, China), bifenthrin (97.0%) from Jiangsu Yangnong Chemical Group Co., Ltd (Jiangsu, China), lambda-cyhalothrin (95.0%) from Jiangsu Huangma Pesticide&Chemical Co., Ltd (Jiangsu, China), deltamethrin (99.0%) and cyfluthrin (92.0%) from Jiangsu Yangnong Chemical Group Corp., Ltd (Jiangsu, China), endosulfan (90.0%) from Jiangsu Rudong Pesticide Co., Ltd (Jiangsu, China), hexaflumuron (95.0%) from East Romble Agrochem (Shan Dong) Co., Ltd (Shandong, China), emamectin benzoate (90.0%) from Zhejiang Qianjiang Biochemical Co., Ltd (Zhejiang, China), abamectin (95.3%) from Shandong Jingbo Agrochemicals Co., Ltd (Shandong, China), fipronil (90.0%) from Anhui Huaxing Chemical Industry Co., Ltd (Anhui, China), imidacloprid (95.0%) from Hubei Sanongda Co., Ltd (Hubei, China), acetamiprid (96.0%) from Qingdao Haili'er Medicine Co., Ltd (Shandong, China), pyridaben (95.0%) from Shandong Sino-Agri United Biotechnology Co., Ltd (Shandong, China). Allelochemicals and insecticides were dissolved in absolute acetone. All other chemicals were of analytical grade and purchased from commercial sources.

Insects

The 1st instar of *M. troglodyta* population was collected from Kunshan, Jinagsu Province, China. They were reared at 26±1ºC, 75% RH and a 12:12 LD photoperiod.

Preparation of cytosolic fractions

Larvae of *M. troglodyta* were homogenized in sodium phosphate buffer (pH 6.5, 0.1M) containing EDTA (1 mM), PMSF (1 mM, dissolved in absolute alcohol) and DTT (1 mM) after peritrophic membranes associated midgut contents were removed respectively. The homogenate was centrifuged at 10 000g for 20 min at 4ºC, and the supernatant was used to determine the enzyme activity.

Assay for glutathione S-transferase activity

The glutathione S-transferase activity using CDNB as the substrate was assayed spectrophotometrically according to Habig et al. (1976). The assay mixture contained 1mM CDNB and 1 mM GSH. The assay was initiated by the addition of 50 μL enzyme; the absorbance at 340 nm was monitored for 2 min. Controls without enzyme always accompanied each assay. Enzyme activity was expressed as nmol/min at 25ºC, and the specific activity as nmol/min/mg protein using an extinction coefficient of 9.6 $m \cdot M^{-1} \cdot cm^{-1}$. The method of Bradford (1976), with BSA as a standard, was used for protein quantitation.

Inhibition of insecticides and allelochemicals on GSTs

Inhibition of CDNB-conjugating activity of GSTs was determined in assays containing 1 mM CDNB and 1 mM GSH and various insecticides (0.01 mM) or allelochemicals (0.01 mM) as inhibitors. All assays, including controls, contained 1% acetone. All assays were run in triplicate.

Statistical analysis

The results were analyzed by analysis of variance (ANOVA) with a level of significance at $p<0.05$.

Results

Enzyme preparations of different instars of *M. troglodyta* larvae were assayed using GSH and CDNB as substrates, and the results were shown in Fig. 1. The 2nd instar represented the highest GST activity among the instars determined. Besides the 2nd instar larvae, the activity of GSTs in the 1st, 3rd, 4th and 5th instar larvae showed a descending order in *M. troglodyta*, and significant differences were observed among them.

The kinetics constants of GSTs for GSH and CDNB were also determined in different instars from *M. troglodyta* larvae. As showed in Table 1, there was a difference in the K_m and V_{max} values of GSTs in different instars. There was no difference in the K_{mCDNB} values of GSTs in different instars ($p>0.05$). Furthermore, the K_{mGSH} values of GSTs in the 1st, 2nd, 3rd and 5th instar larvae showed no significant differences ($p>0.05$). Similarly, there was no difference in the K_{mGSH} values in the 1st and 4th instar ($p > 0.05$). The $V_{maxCDNB}$ value in the 2nd instar was higher than the 5th instar ($p<0.05$), and the $V_{maxCDNB}$ values in 1st, 3rd and 4th instar were no significant differences from the both 2nd and 5th instar ($p>0.05$). Furthermore, the 1st and 2nd instar larvae represented the highest V_{maxGSH} values of GSTs among various instars determined, and no significant differences were observed among them ($p>0.05$), and the V_{maxGSH} values of GSTs in the 4th and 5th instar larvae were the lowest, and no significant differences were observed among them ($p>0.05$).

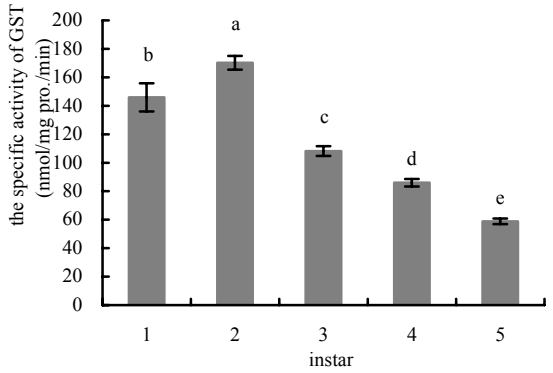

Fig. 1 The specific activity of GST in different instars of *M. troglodyta* larvae

Table 1. The K_m and V_{max} of GSTs in different instars of *M. troglodyta* larvae*

In-star	K_{mCDNB} (mM)	$V_{maxCDNB}$ nmol/mg pro./min	K_{mGSH} (mM)	V_{maxGSH} nmol/mg pro./min
1	2.77±0.87 a	583.93±121.66 ab	0.74±0.072 ab	265.41±8.77 a
2	2.75±0.70 a	652.61±109.33 a	0.89±0.20 a	297.91±24.07 a
3	3.45±2.51 a	603.21±297.03 ab	0.75±0.031 a	190.88±2.69 b
4	3.82±2.09 a	460.50±181.34 ab	0.56±0.09 b	144.64±7.19 c
5	1.53±0.35 a	150.99±19.22 b	0.95±0.10 a	116.13±4.42 c

*Values are means ± standard deviations of the mean of three separate preparations; Means within a column followed by the same small letter are not significantly different.

Table 2. Inhibition of GSTs by insecticides and allelochemicals in various instars of *M. troglodyta* larvae

Inhibitors (0.01mM)	% Inhibition (means±SD)									
	1st instar		2nd instar		3rd instar		4th instar		5th instar	
Organophosphate										
Triazophos	33.71±1.77 bc	B	24.57±2.23 bcd	C	5.82±1.74 efghi	D	9.62±2.17 hi	D	41.12±1.60 bc	A
Malathion	16.52±2.54 efg	B	3.62±1.34 jk	D	8.86±1.32 defg	C	15.20±2.45 ghi	B	32.69±1.45 def	A
Chlorpyrifos	45.31±3.09 a	A	17.64±4.07 efg	B	38.86±3.42 a	A	36.82±3.77 bc	A	40.74±2.11bc	A
Phoxim	19.87±5.11 def	C	-1.34±0.33 kl	D	23.67±3.74bc	C	39.19±3.75 b	B	57.22±1.97a	A
Omethoate	3.24±1.42 h	C	11.42±3.67 ghi	B	-4.24±0.27 ij	D	7.32±0.30 ij	BC	24.78±2.98 fgh	A
Profenofos	16.52±3.69 efg	C	27.87±1.19 b	B	17.47±1.91 cd	C	27.62±1.51 cdef	B	39.85±1.76 bcd	A
Carbamate										
Methomyl	20.76±3.16 de	A	-8.35±1.09 m	C	-2.34±1.88 hi	B	23.29±2.06 defg	A	18.26±1.97 hi	A
Pyrethriod										
Fenpropathrin	7.14±2.54 gh	C	14.65±0.98 efghi	B	4.30±0.54 fghi	C	14.09±1.28 ghi	B	23.88±3.10 gh	A
Beta-cypermethrin	-22.88±4.26 i	D	18.27±2.83 def	B	-15.32±1.75 k	C	34.73±2.74 bc	A	34.23±1.23 cde	A
Bifenthrin	10.05±1.69 fgh	D	17.80±1.64 efg	C	-13.29±1.75 jk	E	31.10±1.74 bcd	A	25.67±2.33 fgh	B
Lambda-cyhalothrin	43.08±2.41 ab	A	12.76±0.27 fghi	C	41.52±2.01 a	A	40.03±2.06 ab	A	28.35±2.33 efg	B
Cyfluthrin	27.46±6.00 cd	A	9.13±1.09 ij	B	24.05±4.56 bc	A	18.97±3.89 efgh	AB	24.90±2.65 fgh	A
Deltamethrin	18.30±4.18 def	A	-6.30±2.00 lm	B	14.18±2.15 cdef	A	17.15±1.78 fghi	A	18.26±0.96 hi	A
Organochlorine										
Endosulfan	34.82±4.84 abc	A	26.46±1.91 bc	AB	19.62±2.23 c	B	38.08±7.30 bc	A	38.95±5.10 bcd	A
Other insecticides										
Hexaflumuron	-19.20±4.64 i	B	16.06±1.91 efgh	A	15.51±6.18 cde	A	15.20±1.58 ghi	A	6.70±2.44 jk	A
Emamectin benzoate	-34.82±5.58 j	A	-83.94±4.39 n	D	-83.04±4.31 l	CD	-71.27±6.39 l	C	-53.64±1.01 m	B
Abamectin	41.74±2.01 ab	A	10.87±1.66 hi	C	23.48±5.10 bc	B	-38.63±8.58 k	E	-14.56±1.33 l	D
Fipronil	40.85±1.55 ab	A	28.66±1.64 b	B	-1.01±1.07 ghi	E	18.55±0.87 efgh	C	6.26±0.80 jk	D
Imidacloprid	12.50±3.37 efgh	A	2.91±0.33 jk	B	-1.77±0.54 hi	C	15.20±0.97 ghi	A	0.96±0.27 k	BC
Acetamiprid	5.58±1.89 h	C	20.94±1.19 cde	A	6.58±1.61 efgh	C	-2.51±0.59 j	D	12.64±0.54 ij	B
Pyridaben	35.49±1.39 abc	A	-2.28±1.00 klm	C	17.97±3.95 cd	B	13.81±2.96 ghi	B	36.27±4.06 cde	A
Allelochemical										
Quercetin	34.15±0.77 bc	B	46.93±1.97 a	A	32.66±6.85 ab	B	49.93±1.69 a	A	44.96±3.76 b	A
Tannic acid	17.97±2.37 def	B	41.26±3.67 a	A	20.38±4.96 c	B	28.17±2.45 cde	B	18.39±4.88 hi	B
2-tridecanone	18.30±1.77 def	A	15.91±1.25 efgh	AB	20.76±3.23 c	A	10.04±2.37 hi	B	15.20±1.97 i	AB

The data were analyzed using analysis of variance (ANOVA). The difference is significant if $p<0.05$.;Means within a column followed by the same small letter are not significantly different; Means within a row followed by the same capital letter are not significantly different.

The inhibition of GST activity of different instars by insecticides and allelochemicals is shown in Table 2. For the 1st instar, organophosphate insecticides (chlorpyrifos), pyrethriod insecticides (lambda-cyhalothrin), organochlorine (endosulfan) and other insecticides (abamectin, fipronil and pyridaben) were the best inhibitors tested. The inhibiting effect of allelochemicals

(quercetin) was better, inhibiting more than 34% of GSTs activity at a concentration of 0.01 mM. For the 2nd instar, two allelochemicals (tannic acid and quercetin) were the most potent inhibitors tested, inhibiting more than 40% of GST activity at a final concentration of 0.01 mM. For the 3rd instar, the inhibitory effects of quercetin, organophosphate insecticides (chlorpyrifos) and pyrethriod insecticides (lambda-cyhalothrin) were the highest. The inhibitory effects of quercetin and lambda-cyhalothrin were the highest for the 4th instar larvae. Furthermore, for the 5th instar, the inhibitory effect of phoxim was the highest. This suggests that GSTs from different instar are qualitatively different in isozyme composition and thus different in sensitivity to inhibitors.

Discussion

Glutathione S-transferases belong to phase II detoxification system involved in conjugation reactions and may also detoxify a number of toxic ligands by acting as a non-catalytic intracellular binding protein (Kostaropoulos et al. 2001). It is believed that these enzymes play essential roles in the survival of insects exposed to endogenous or exogenous xenobiotics.

The significant differences in GST activity and kinetic characteristics observed among the 5 instars imply that the GSTs were different in different instars. The multiple isoenzyme hypothesis may explain our results. Multiple GSTs isoenzymes are a common phenomenon in vertebrates, like fish and mammals. The presence of multiple isoenzymes is common in insects and has been reported in a number of species. In *Tenebrio molitor* Linnaeus larvae, the existence of 4 isoenzymes was reported, as well as alternations in some GST characteristics during development (Kostaropoulos et al. 1996). In fall armyworm, *Spodoptera frugiperda* (J. E. Smith), the midgut possesses 5 isoenzymes, namely MG GST-1, MG GST-2, MG GST-3, MG GST-4 and MG GST-5, all of which are heterodimers with subunit molecular weights of 26 700 to 30 000, but no qualitative difference in isoenzyme composition is observed during larval development (Yu 1995). Therefore, our findings reveal a very interesting developmental model of GSTs that is followed by *M. troglodyta* larvae, to our knowledge, is unique. Furthermore, the multiple GSTs isoenzymes in various instars of *M. troglodyta* larvae are required to be confirmed.

In a previous investigation, Smirle (1993) found a positive correlation between the activity of the detoxification enzymes GSTs and the resistance to certain insecticides. Thus, controlling the activity of these enzymes would be highly useful for management pests through different ways. Therefore, *in vitro* inhibition of GST activity by insecticides and allelochemicals in various instars of *M. troglodyta* larvae was studied. These results may contribute to the understanding of the difference in sensitivity of different instars to pesticides, and could provide the basis for integrated pest management of *M. troglodyta.*

In our study, the inhibitory effects of the few organophosphorous insecticides tested were higher than other insecticides, and carbamate and pyrethroids insecticides were moderately inhibitory to GST activity. However, the inhibitory effect of lambda-cyhalothrin was higher than other pyrethroids. Chen et al. (2007) reported that organophosphates and carbamates are important substrates for GSTs. Although it was generally accepted that pyrethroids were not the substrate of glutathione S-transferase, it has been reported that they bind with GST or the active site of the enzyme (Kostaropoulos et al. 2001). But GSTs in different instars are qualitatively different in isozyme composition and, thus, different in sensitivity to insecticides (Table 2). For example, for GST activity in the 5th instar, phoxim was the best inhibitors tested, inhibiting ＞55% of GST activity at a concentration of 0.01mM. But for the activity in the 1st and 3rd instar, chlorpyrifos was the best inhibitor. These results suggest that isoenzymes of GSTs may affect susceptibility to insecticides.

There are several reports that naturally-occurring polyphenols are potent inhibitors of GST activity in insects (Wadleigh&Yu 1987, Wadleigh & Yu 1988, Yu& Abo-Elghar 2000, Chen et al. 2007). Quercetin are plant phenols, and potent inhibition was observed in our study. Yu and Abo-Elghar (2000) suggested that some allelochemicals found in certain foliage might serve as synergists by interfering with GST-mediated detoxification in phytophagous insects. In this study, we found that quercetin was the most effective inhibitor of GSTs from *M. troglodyta*. However, the application of quercetin as a synergist of insecticides is scarely reported, which needs more study on its *in vivo* effects on GSTs and/or other degradation enzymes and its interaction with insecticides *in vivo*. Therefore, our results demonstrate that quercetin had the potential of serving as a synergist for enhancing the toxicity of insecticides in *M. troglodyta*. However, its application as a synergist in insect control requires further investigation.

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